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METHOD OF AMPLIFICATION OF NUCLEIC ACID SEQUENCE AND REAGENT KIT USED

[kakusanhairetsu no zohfukuhoho oyobi sonotame no kitto]

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[There are no amendments to this patent.]

(54) [Title of the invention]

Method of amplification of nucleic acid sequence and reagent kit used

(57) [Abstract]

[Objective] Amplification of a nucleic acid sequence can be achieved using a simple method.

[Constitution] A cyclic single-chain nucleotide (A), a primer (B) having at least a partially complementary sequence with said nucleotide (A) or a fragmented target nucleic acid sequence (D), and a heat-resistant polymerase (C) are used, and primer (B) is stretched and annealed with cyclic single-chain nucleotide (A) to form a single-chain nucleic acid sequence having a repeated sequence complementary to cyclic single-chain nucleotide (A).

[Claims of the invention]

[Claim 1] A method of amplification of a nucleic acid sequence wherein a cyclic single-chain nucleotide (A), a primer (B) having at least a partially complementary sequence with said nucleotide (A) or a fragmented nucleic acid sequence (D), and a heat-resistant polymerase (C) are used, and the primer (B) is stretched and annealed with cyclic single-chain nucleotide (A) to form a single-chain nucleic acid sequence having a repeated sequence complementary to the cyclic single-chain nucleotide (A).

[Claim 2] A method of amplification of a nucleic acid sequence wherein a target nucleic acid sequence (D) in a test sample and a cyclic single-chain nucleotide (A) having a partially complementary sequence with at least the target nucleic acid sequence (D), and a heat-resistant DNA polymerase (C) are used, the target nucleic acid sequence (D) annealed with the cyclic singe-chain nucleotide (A) is used as a primer, and a sequence having a sequence complementary to the cyclic single-chain nucleotide (A) is produced.

[Claim 3] A method of amplification of a nucleic acid sequence wherein a cyclic sequence singe-chain nucleotide (A), a primer (B) having at least a partially complementary sequence and a heat resistant DNA polymerase (C) are used, and procedures (a) through (d) below are carried out.

Procedure (a): Annealing is performed for the cyclic single-chain nucleotide (A) and primer (B).

Procedure (b): The cyclic single-chain nucleotide (A) is used as a template, and annealed primer (B) is stretched and amplification of nucleic acid sequence is achieved.

Procedure (c): the amplified nucleic acid sequence is fragmented.

Procedure (d): The fragmented nucleic acid sequence is used as a primer, as needed, and the

procedures (a) through (c) above are repeated at least one time.

[Claim 4] A method of amplification of a nucleic acid sequence wherein the target nucleic acid sequence (D) included in the test, and cyclic single-chain nucleotide (A) having at least a partially complementary sequence with the above-mentioned target nucleic acid sequence (D), and a heat-resistant DNA polymerase (C) are used, and procedures (a) through (e) below are carried out.

Procedure (a): The target nucleic acid sequence (D) is fragmented.

Procedure (b): The fragmented target nucleic acid sequence (D) and cyclic single-chain nucleotide (A) are annealed.

Procedure (c): The cyclic single-chain nucleotide (A) is used as a template, and the annealed target nucleic acid sequence (D) is stretched with a heat resistant DNA polymerase (C) and stretching is carried out with the annealed and fragmented target nucleic acid sequence (D) as a primer, and amplification of nucleic acid sequence is achieved.

Procedure (d): nucleic acid sequence is fragmented.

Procedure (e): The fragmented nucleic acid sequence is used as a primer, as needed, and processes (a) through (d) above are repeated at least one time.

[Claim 5] The method of amplification of a nucleic acid sequence nucleic acid sequence specified in claims 1 through 4 wherein the heat-resistant DNA polymerase is a polymerase having a helicase-like activity.

[Claim 6] When the heat-resistant DNA polymerase is reacted at a high temperature using a cyclization DNA as a template, the amplification method for nucleic acid sequence specified in

claims 1 through 5 above wherein a polymerase having a helicase-like activity is a polymerase capable of continuously synthesizing DNA as the DNA previously synthesized DNA is peeled off as a single cycle of synthesis is completed.

[Claim 7] A reagent kit used for the method of amplification of a nucleic acid sequence that includes a cyclic single-chain nucleotide (A) having at least a partially complementary sequence with the target nucleic acid (D) included in the test sample, heat resistant DNA polymerase (C) and deoxyliponucleotide triphosphoric acid.

[Claim 8] The reagent kit used for the method of amplification of a nucleic acid sequence specified in claim 7 above wherein the heat-resistant DNA polymerase is a polymerase having a helicase-like activity.

[Claim 9] The reagent kit used for the method of amplification of a nucleic acid sequence specified in claim 7 above wherein, when the heat-resistant DNA polymerase is reacted at a relatively high temperature using a cyclization DNA as a template, the polymerase having a helicase-like activity is a polymerase capable of continuously synthesizing DNA as the DNA previously synthesized is peeled off as a single cycle of synthesis is completed.

[Detailed explanation of the invention]

[0001]

[Field of industrial application] The present invention pertains to a method of amplification of a nucleic acid sequence and a reagent kit used. In particular, the present invention pertains to a method capable of producing a greater quantity of nucleic acid produced by a base sequence compared with the initial amount. When the method of the present invention is used, diagnosis of hereditary diseases, cancers, and infectious diseases becomes easier.

[0002]

[Prior art] In recent years, detection of nucleic acids via the hybridization method is being widely used as an effective means for diagnosis of hereditary diseases, cancers, and infectious diseases. In the nucleic acid detection method, the target base sequence is a very small portion of the nucleic acid, in some cases, and when a detection method wherein a nonradioactive target probe or an oligonucleotide wherein the end is marked with a radioactive isotope is used, detection is difficult due to insufficient sensitivity. Much research has been done in the area of improving sensitivity of the probe detection system (for example, WO87/03622, etc.). Also, as a means to increase the sensitivity, a method wherein the target nucleic acid is amplified with a DNA polymerase is disclosed (Japanese Kokai Patent Application No. Sho 61[1986]-274697, hereinafter, "PCR" in some cases). However, a complex temperature control is required in the above-mentioned method, and special equipment is required. An amplification method where a DNA ligase is used also has been disclosed (for example, WO89/12696, Japanese Kokai Patent Application No. Hei 2[1990]-2934, etc.). However, when said method is used, non-singular amplification occurs due to the blunt end ligation of the DNA ligase. As a means to avoid the

above-mentioned problem, at least three pairs of probes are used in WO89/12696, but because of the number of probes involved, cost is a problem. Also, production of RNA from DNA using an RNA polymerase is known, and a method wherein amplification of the nucleic acid with an RNA polymerase is disclosed (WO89/01050). However, when the above-mentioned method is used, adequate amplification is not possible when transfer amplification based on the RNA polymerase alone is used. For this reason, a further reaction is carried out for the RNA produced with a reverse transfer enzyme, and DNA is produced. Meanwhile, a method wherein amplification is done with a hybridization probe alone after hybridization is of the target nucleic acid with the probe is also known (*Biotechnology* vol. 6, 1197, 1988). However, in said method, the probe connected by the non-singular reaction is also amplified, and an increase in the blank value occurs.

[0003]

[Problems to be solved by the invention] The objective of the present invention to provide a method of amplification of a nucleic acid sequence of a target nucleic acid.

[0004]

[Means to solve problems] As a result of their continuous effort to eliminate the above-mentioned problems, the inventors discovered that, when the heat-resistant DNA polymerase is reacted at a relatively high temperature using a cyclization DNA as a template, a certain type of heat-resistant DNA polymerase is capable of continuous synthesis of DNA as the DNA previously synthesized is peeled off as a single cycle of synthesis is completed. Based on the above background, it was also discovered that the above-mentioned problems can be eliminated when a cyclic single-chain nucleotide (A) having at least a partially complementary sequence

with the target nucleic acid (D) and a heat resistant DNA polymerase (C) are used, and the result is the present invention.

[0005] In other words, the present invention is a method of amplification of a nucleic acid sequence wherein a cyclic single-chain nucleotide (A), a primer (B) having at least a partially complementary sequence with said nucleotide (A) or a fragmented nucleic acid sequence (D), and a heat-resistant polymerase (C) are used, and the primer (B) is stretched and annealed with the cyclic single-chain nucleotide (A) to form a single-chain nucleic acid sequence having a repeated sequence complementary to the cyclic single-chain nucleotide (A). Furthermore, the present invention is a reagent kit used for the method of amplification of a nucleic acid sequence that includes a cyclic single-chain nucleotide (A) having at least a partially complementary sequence with a target nucleic acid (D) included in the test sample, a heat resistant DNA polymerase (C), and deoxyliponucleotide triphosphoric acid.

[0006] The length and the sequence of the cyclic single-chain nucleotide (A) used in the present invention is not especially limited. Also, a synthetic nucleic acid, or a natural nucleic acid based on virus DNA or plasmid DNA can be used. When a target nucleic acid sequence (D) is used as a primer, the cyclic single-chain nucleotide (A) is one having the length of the annealed portion complementary with the target nucleic acid sequence (D) in the range of 10~40 nucleotides. Also, in general, the size is in the range of 50~10000 nucleotides, and 100~5000 nucleotides is especially desirable.

[0007] The length and the sequence of the primer (B) having at least a partially complementary with the cyclic single-chain nucleotide (A) used in the present invention are not limited, as long as at least a portion of the sequence is complementary with the cyclic single-chain nucleotide

(A). Furthermore, either a synthetic nucleic acid, or a natural nucleic acid can be used. The portion complementary with the cyclic single-chain nucleotide (A) has a length in the range of 6~100 nucleotides, and 10~40 nucleotides is especially desirable.

[0008] When the heat-resistant DNA polymerase is reacted at a relatively high temperature using a cyclization DNA as a template, the type of polymerase used is not especially limited as long as a polymerase with a helicase-like activity is a polymerase capable of continuously synthesizing DNA as the DNA previously synthesized is peeled off as a single cycle of synthesis is completed. For example, Tth (Thermus thermophilus) DNA polymerase (*Eur. J. Biochem.*, 149, 41, (1985)), DNA polymerase (*J. Bacteriol.*, 127, 1550, (1976), Vent (Thermococcus litoralis) DNA polymerase, Bst (Bacillus sterothermophilus) DNA polymerase, etc. can be used. Furthermore, enzymes having a helicase-like activity such as ϕ29 DNA polymerase, and M2 DNA polymerase can also be used despite a lack of heat-resistant. The temperature used for the reaction varies depending on the type of enzyme used, and in general, is in the range of 40°C~90°C, but a temperature in the range of 60°C~80°C is especially suitable.

[0009] As for the target nucleic acid (D) used in the present invention, the length and the structure of the substance is not especially limited, as long as it can be fragmented and can be used as a primer for the DNA polymerase (C).

[0010] In the nucleic acid amplification method of the present invention, a cyclic sequence singe-chain nucleotide (A), a primer (B) having at least a partially complementary sequence and a heat resistant DNA polymerase (C) are used, and procedures (a) through (d) described below are carried out.

Procedure (a): An annealing is performed for the cyclic single-chain nucleotide (A) and the

above-mentioned primer (B).

Procedure (b): The cyclic single-chain nucleotide (A) is used as a template, and annealed primer (B) is stretched and amplification is performed for the nucleic acid sequence.

Procedure (c): the amplified nucleic acid sequence is fragmented. Procedure (d): The above-mentioned fragmented nucleic acid sequence is used as a primer, as needed, and the above-mentioned procedures (a) through (c) are repeated at least one time.

[0011] Also, in the method of amplification of a nucleic acid sequence of the present invention, the target nucleic acid sequence (D) in the test sample and cyclic single-chain nucleotide (A) having at least a partially complementary sequence with the above-mentioned target nucleic acid sequence (D), and a heat-resistant DNA polymerase (C) are used, and procedures (a) through (e) below are carried out.

Procedure (a): The target nucleic acid sequence (D) is fragmented.

Procedure (b) The fragmented target nucleic acid sequence (D) and the above-mentioned cyclic single-chain nucleotide (A) are annealed.

Procedure (c): The cyclic single-chain nucleotide (A) is used as a template, and the annealed target nucleic acid sequence (D) is stretched with heat resistant DNA polymerase (C) and the annealed and fragmented target nucleic acid sequence (D) as a primer is stretched, and amplification is performed for the nucleic acid sequence.

Procedure (d): The nucleic acid sequence produced is fragmented.

Procedure (e): The above-mentioned fragmented nucleic acid sequence is used as a primer, as needed, and the above-mentioned procedures (a) through (d) are repeated at least one time.

[0012] The present invention is explained further with reference to Fig. 1. It should be noted that

in the figure, A is the cyclic single-chain nucleotide, B is the primer, and C is the heat-resistant DNA polymerase.

Procedure (a): The cyclic single-chain nucleotide (A) and primer (B) are annealed. Annealing is performed at a temperature selected to provide maximum selectivity. In general, the treatment is carried out as the temperature is increased in such a manner that a singular bond can be achieved for the cyclic single-chain nucleotide (A) and primer (B) and non-singular bond based on mismatches can be minimized.

Procedure (b): The cyclic single-chain nucleotide (A) is used as a template, and the annealed primer (B) is stretched and amplification is of the nucleic acid sequence is achieved. The above-mentioned treatment can be achieved by stretching dNTP (four types of deoxyliponucleotide triphosphoric acids consisting of dATP, dCTP, dGTP, and dTTP) and a heat-resistant DNA polymerase (for example, Thermus thermophilus DNA polymerase, Thermus aquaticus DNA polymerase, etc.) using the cyclic nucleotide as a template. When a reaction is carried out at a high temperature with the above-mentioned enzymes, the double-chain portion of the DNA can be peeled off, and synthesis of the primer can be continued.

Procedure (c): The amplified nucleic acid sequence is fragmented.

Procedure (d): The above-mentioned fragmented nucleic acid sequence is used as a primer, as needed, and the above-mentioned procedures (a) through (c) are repeated at least one time.

[0013] The present invention is explained further with reference to Fig. 2. It should be noted that in the figure, A is the cyclic single-chain nucleotide, D is the target nucleic acid sequence, and C is the heat-resistant DNA polymerase.

Procedure (a): The target nucleic acid sequence (D) is fragmented.

Procedure (b): The fragmented target nucleic acid sequence (D) and the above-mentioned cyclic single-chain nucleotide (A) are annealed.

Procedure (c): The cyclic single-chain nucleotide (A) is used as a template, and the annealed target nucleic acid sequence (D) is stretched with heat resistant DNA polymerase (C) and the annealed and fragmented target nucleic acid sequence (D) as a primer is stretched, and the nucleic acid sequence is amplified.

Procedure (d): The nucleic acid sequence produced is fragmented.

Procedure (e): The above-mentioned fragmented nucleic acid sequence is used as a primer, as needed, and the above-mentioned procedures (a) through (d) are repeated at least one time.

[0014]

[Effect of the invention] According to the amplification method of the present invention, multiple nucleic acid sequences can be produced from a single molecule of a cyclic single-chain nucleotide when a heat-resistant DNA polymerase having a helicase-like activity is used; thus, efficient amplification can be achieved. Furthermore, the amplification method of the present invention does not require a complex temperature control system or special equipment. Furthermore, the amplification method of the present invention is not a method used for amplification of the probe; thus, amplification of the residual probe based on mis-matches or non-singular hybridization is absent, and the S/N (Signal/Noise) ratio can be increased.

[0015]

[Application examples] In the following, the effect of the present invention is explained further with an Application example of the present invention and a comparative example, but the

present invention is not limited to these examples.

Application Example 1

A single-chain Brucescript [bluescript?, phonetic] KS+DNA purified as usual was used as the cyclic single-chain nucleotide (A) and M13 primer P1 (product of Toyoboseki Corp.) was used as primer (B) and the procedures shown below were carried out.

Procedure (a)

1 pmol of cyclic single-chain nucleotide (A) and 10 pmol of primer (B) were added to 20 μl of reaction solution. It was then insulated at 94°C for 2 minutes; then, further insulated at 50°C for 5 minutes, and annealing was performed.

Reaction solution

10 mM	Tris-HCl (pH. 8.9)
1.5 mM	$MgCl_2$
80 mM	KCl
500 μg/ml	BSA
0.1%	Sodium cholate
0.1%	Trinton X-100
2 mM	dATP, dGTP, dCTP, and dTTP

Procedure (b)

4 units of Tth DNA polymerase (product of Toyoboseki Corp.) was added to the above-mentioned reaction solution, the temperature was retained at 75°C for 60 minutes, and stretching of the primer was carried out.

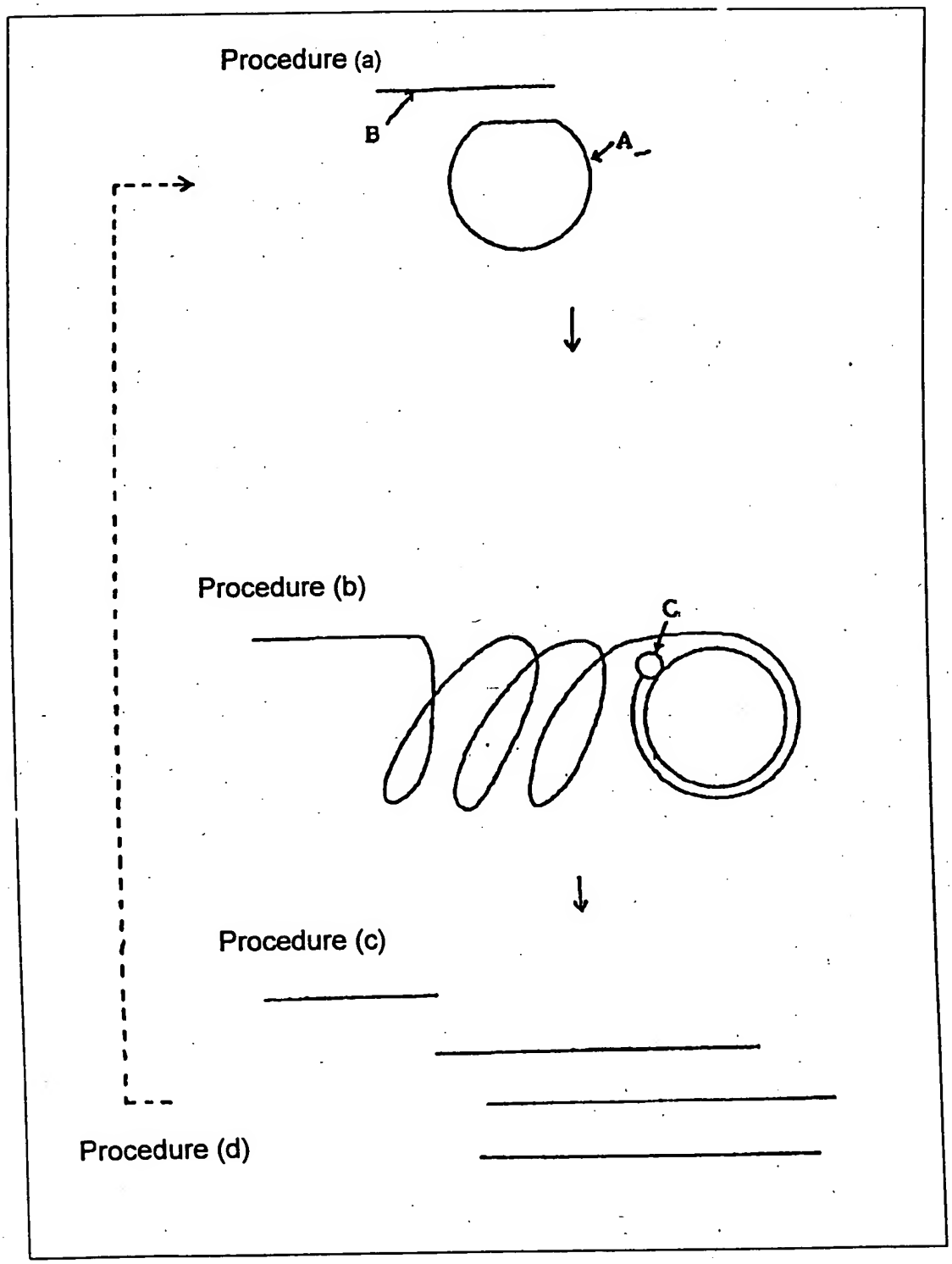
Procedure (c)

Subsequently, cataphoresis was performed with an agarose gel, and the DNA synthesis was confirmed by the ethydium bromide dyeing method. The results indicated that the cyclic single-chain nucleotide (A) of the template alone was observed 0 min (lane 2) after the start of the reaction, but synthesis of DNA was observed at the higher molecular weight side than that of the template after insulation for 30 minutes (lane 3), and for 60 minutes (lane 4). This indicates that primer (B) was stretched with cyclic single-chain nucleotide (A) as a template, and amplification of the nucleic acid sequence was performed.

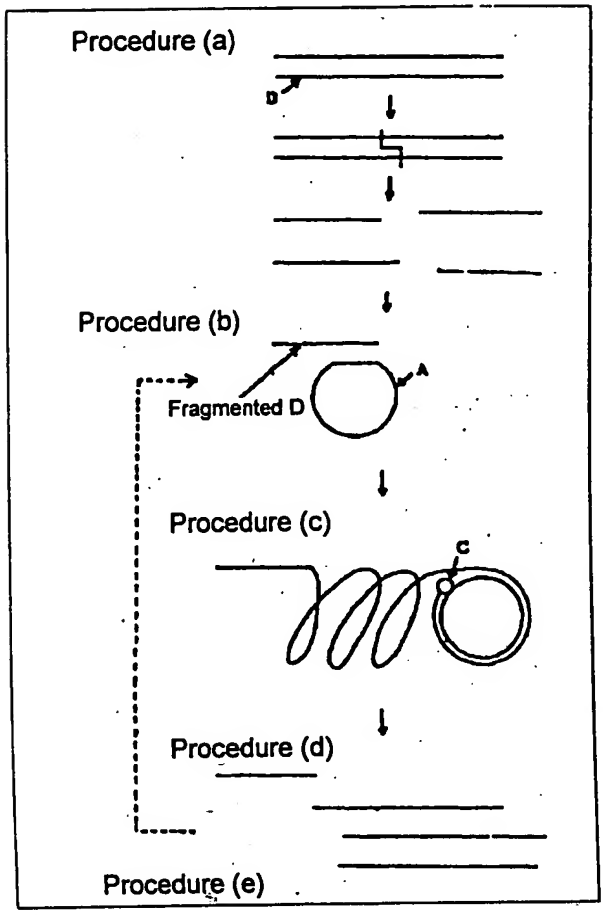
[Brief description of figures]

Fig. 1 shows a diagram that demonstrates of the principle of the present invention. In the figure, A represents the cyclic single-chain nucleotide, B represents the primer, and C represents the heat-resistant DNA polymerase. Fig. 2 shows a diagram that demonstrates the principle of the present invention. In the figure, D represents a target nucleic acid sequence, A represents the cyclic single-chain nucleotide, B represents the primer, and C represents the heat-resistant DNA polymerase. Fig. 3 shows the cataphoresis pattern of the DNA synthesized in Application Example 1. Lane 1 corresponds to the marker DNA (λ-Hind III), [lanes] 2, 3, and 4 correspond to patterns after insulation for 0 minutes, 30 minutes, and 60 minutes, respectively, at a temperature of 75°C. The arrow indicates the position of the cyclic single-chain nucleotide (A) used as the template.

[Fig. 1]



[Fig. 2]


Procedure (a)
D
Procedure (b)
Fragmented D
A
Procedure (c)
C
Procedure (d)
Procedure (e)

[Fig. 3]